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Conservation of holm oak (*Quercus ilex*) by in vitro culture

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Abstract. *In vitro* culture techniques are used to propagate tree species, as well as to conserve the species in the short and long term. In the present study, *in vitro* propagation and conservation of holm oak (*Quercus ilex* L.) were successfully achieved using juvenile material. Mature acorns were germinated under controlled conditions of moisture and temperature, and 3-month-old seedlings were used as source of explants for culture initiation. Micropropagation via axillary bud proliferation was achieved by culturing shoots in a vertical position on Woody Plant Medium containing different cytokinins and/or concentrations, which were changed every 2 weeks over a 6-week multiplication cycle, as follows: 0.1 mg L^{-1} benzyladenine (BA) for the first 2 weeks, 0.05 mg L^{-1} BA for the next 2 weeks, and 0.01 mg L^{-1} BA plus 0.1 mg L^{-1} zeatin for the last 2 weeks. Acceptable rooting rates were obtained by culturing microcuttings in Murashige & Skoog medium with half-strength macronutrients supplemented with 3 or 5 mg L^{-1} indole-3-butyric acid (IBA) in combination with 0.1 mg L^{-1} naphthalene acetic acid (NAA) for 15 days and subsequent transfer to auxin-free medium for 4 weeks.
Keywords: Axillary shoot proliferation; *In vitro* conservation; Micropropagation; *Phytophthora cinnamomi*; Seedlings.

Conservación de *Quercus ilex* mediante cultivo in vitro

Resumen. Las técnicas de cultivo *in vitro* permiten la propagación de especies leñosas, así como su conservación a corto y largo plazo. En este trabajo se logró, con éxito, la propagación y la conservación *in vitro* de encina (*Quercus ilex* L.) partiendo de material juvenil. Los explantos utilizados para el establecimiento *in vitro* se obtuvieron a partir de plántulas de 3 meses procedentes de bellotas germinadas bajo condiciones controladas de humedad y temperatura. Los brotes establecidos *in vitro* se micropropagaron vía proliferación de yemas axilares cultivándolos en posición vertical en medio Woody Plant Medium durante 6 semanas con transferencias a medio fresco cada 2 semanas donde se modifica el tipo de citoquinina y su concentración, como sigue: 0,1 mg L^{-1} benciladenina (BA) durante las 2 primeras semanas, transferencia a 0,05 mg L^{-1} BA las 2 semanas siguientes, y por último se transfieren a 0,01 mg L^{-1} BA más 0,1 mg L^{-1} zeatina durante 2 semanas más, hasta completar las seis semanas. Las tasas de enraizamiento fueron aceptables cuando los brotes se cultivaron en medio Murashige & Skoog con los macronutrientes reducidos a la mitad adicionado con 3 ó 5 mg L^{-1} de ácido indol-3-butírico (AIB) en combinación con 0,1 mg L^{-1} de ácido naftalenacético (ANA) durante 15 días y posterior transferencia al mismo medio sin auxinas durante 4 semanas.
Palabras clave: Proliferación vía yemas axilares; conservación *in vitro*; Micropropagación; *Phytophthora cinnamomi*; plántulas.

Introduction

Quercus ilex L. (*Q. ilex* subsp. *ballota*, syn. *Q. rotundifolia* and *Q. ilex* subsp. *ilex*) is the one of most representative tree in Mediterranean forests (Marañón, 1988). The species is distributed along the Mediterranean Basin, in Portugal, Spain, Southern France, Sardinia, Algeria, Morocco and Tunisia (Anon., 2007). In the Iberian Peninsula, the species occupies an area of almost four million hectares, mainly in Spanish territory (Pinto-Correia & *al.*, 2011). Well conserved holm oak woodlands constitute one of the most complex and mature natural ecosystems in the region. Human intervention during the last few centuries has transformed these ecosystems into wooded pasture land with scarce bush cover, forming a savannah-like landscape known as *dehesa* (Bellido, 1996) –considered the paradigm of sustainable agrosilviculture– and included as a special conservation area in Council Directive 92/43/EEC (Anon., 1996). The vegetation in *dehesa* comprises a tree layer of Mediterranean evergreen oaks (*Q. ilex* and *Q. suber*) of relatively slow growth and speed of regeneration and with an important stabilizing and diversifying function; when mixed with pastures, the oaks display a higher growth rate and speed of regeneration (San Miguel Ayanz, 1994). The diversity of production (forage, acorn, wood, cork or charcoal) converts this type of woodland into an agro-sylvo-pastoral system of great ecological and economic importance. Production of holm oak acorns is essential for feeding wildlife and fattening porcine livestock (Ruíz de la Torre, 2006). The species also establishes symbiotic relationships with several edible fungi such as some species of the *Tuber* genus, among which the black truffle (*T. melanosporum*) is the most highly appreciated in haute cuisine. These

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factors, together with the use of the wood for fuel and construction, contribute significantly to rural development and to stabilization of the population in economically depressed areas (Reyna-Domenech & García Barreda, 2008). The high landscape and recreational value of the ecosystem also contributes to the development of a new economic activity, namely rural tourism (Serrada-Hierro & San Miguel, 2008).

Oak decline syndrome affects oaks worldwide and is of great concern. Although the decline was first observed at the start of the 20th century, *Q. ilex* was not severely affected until around 30 years ago (Sánchez & *al.*, 2002; Sampaio & *al.*, 2012; Corcobado & *al.*, 2014). Oak decline, in Spanish *la seca*, is a disease of complex aetiology, caused by the combined action of a variable number of biotic and abiotic factors that may lead to the sudden death of affected trees or gradual decline of the trees until their death (Sampaio & *al.*, 2012). Symptoms of holm oak decline are very unspecific and include wilting and leaf chlorosis, loss of foliage, branch lesions, sprouting of epicormic shoots, root rot and trunk exudations. The oomycete *Phytophthora cinnamomi* has been considered the main biotic cause of holm oak decline in Spanish *dehesas* (Corcobado & *al.*, 2013). The action of *P. cinnamomi* may be favoured by a series of abiotic factors, such as soil moisture content (severe recurring drought and seasonal flooding), edaphic factors (shallow soils, soil compaction and fine texture of soils), changes in the traditional use of the *dehesas*, and biotic factors, such as the presence of defoliating insects and pathogens such as *Botryosphaeria* spp. (*Diplodia* spp.) and *Biscogniauxia mediterranea* (*Hypoxylon mediterraneum*), also implicated in the decline. The presence of the bacterium *Brenneria quercina* has also been found to be associated with *Quercus* decline in Spain 14 years ago (Biosca & *al.*, 2003).

Holm oak in the *dehesas* has a low capacity for natural regeneration due to factors such as aging of the trees, the highly variable acorn production, as well as heavy predation of these, limited dispersion and limited conditions for germination and establishment (Plieninger & *al.*, 2004; Corcobado & *al.*, 2013). Measures that enable recovery of the density of trees in affected areas are required as a result of the extent of the decline problem and the low level of natural regeneration of holm oak in the *dehesas*. Selection and vegetative propagation of individual trees that are tolerant to oak decline is one possible solution to the problem. Various studies have cited genetic differences between holm oak populations in relation to the susceptibility/tolerance to attack by *P. cinnamomi* (Navarro & *al.*, 2004; Tapias & *al.*, 2006). The capacity of some individuals to survive in areas strongly affected by the stress associated with oak decline and the results of greenhouse trials in which *Q. ilex* is artificially infected with *P. cinnamomi* together indicate a high degree of genetic variation in the species (Tapias & *al.*, 2006). The vegetative propagation of these tolerant individuals could be a possible alternative, however holm oak is considered recalcitrant to vegetative propagation. Propagation by cuttings is greatly influenced by the age of the ortet, as the rooting rate decreases with the cutting age (L´Helgoual'ch & Espagnac, 1987). A recent review has described forest biotechnology techniques as an emerging opportunity for tree improvement and conservation in *Quercus* species (Vieitez & *al.*, 2012). Micropropagation techniques represent an alternative approach for the rapid propagation and improvement of forest stock, especially when conventional propagation is difficult to achieve (Renau-Morata & *al.*, 2005; Gomes & Canhoto, 2009; San José & *al.*, 2013). *In vitro* culture techniques also allow conservation of species with low seed viability, establishment of cultures for clonal mass propagation, and production of highly heterozygous crops or cultures that must be propagated vegetatively to preserve their genetic integrity (Corredoira & *al.*, 2011). Although the use of *in vitro* methods increases the numbers of individuals, the degree of diversity depends on the genetic diversity of the material available for initiating cultures (Pence, 2010). In this respect, the use of seedling material to establish *in vitro* cultures enables production of large numbers of plants of diverse genotypes necessary for maintaining genetic diversity in forest ecosystems. This fact could minimize the genetic monoculture often associated with "clonal" plantations raised by tissue culture (Ostrolucká & *al.*, 2007; Tamta & *al.*, 2008). Maintenance of genetic diversity and conservation of forest genetic resources are critical to forest sustainability, ecosystem stability and continued adaptation and survival of tree species.

Micropropagation by axillary shoot proliferation may represent a valuable tool for propagating recalcitrant tree species such as holm oak. Previous reports on the micropropagation of holm oak by axillary budding are scarce. To our knowledge, Liñán & *al.* (2011) have reported micropropagation of holm oak via proliferation of shoots obtained from mature acorns, although a clear, reproducible protocol was not provided. Martínez & *al.* (2017a) subsequently described a procedure for the micropropagation of adult holm oak trees by axillary shoot proliferation. Additionally, leaf and shoot apex explants excised from *in vitro* shoot cultures of holm oak have been used to initiate somatic embryogenic cultures (Martínez & *al.*, 2017b,c).

The objective of the present study was therefore to develop an efficient method for the *in vitro* propagation of holm oak seedlings that enables the conservation of multiple genotypes.

Material and methods

Plant material and culture conditions

Mature acorns were harvested in November 2013 from trees growing in Boadilla del Monte (Madrid). The acorns were immersed for 2.5 h in a fungicide solution (3.5 g L^{-1} Cuprosan WG, Bayer, Germany) and were then dried at room temperature for 48h. Half of the acorns were stored for 2 months in darkness at 4ºC in plastic boxes containing sterile sand moistened, whereas the other half was directly germinated. In both cases,

the acorns were placed in moistened perlite in trays and were germinated in a growth chamber at 25ºC and 80-90% relative humidity under a 16 h photoperiod (90-100 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps). Three-month-old seedlings were used as source of explants for micropropagation experiments. The shoots were stripped of leaves and the surfaces were then disinfected by immersing the shoots in a 0.25% solution of free chlorine (Millipore® chlorine tablets; Merck) containing 2-3 drops of Tween 80®, for 3 min. The shoots were then rinsed three times, each of 10 min, in sterile water. Disinfected shoots were sectioned into 0.5 cm shoot tips and nodal segments, which were placed upright in 30 x 150 mm culture tubes (one explant per tube) containing 20 mL of initiation medium. This medium consisted of Woody Plant Medium (WPM, Lloyd & McCown 1980) supplemented with 0.5 mg L^{-1} benzyladenine (BA), 0.5 mg L^{-1}indoleacetic acid, 80 mg L^{-1} ascorbic acid, 30 g L^{-1} sucrose and 6.5 g L^{-1} Vitro agar (Pronadisa, Spain), brought to pH 5.7, and autoclaved at 115ºC for 20 min. To avoid contact with excreted phenolic compounds, each explant was moved to the opposite side of the culture tube 1-day after culture initiation. The explants were transferred to fresh initial medium every two weeks until 8 weeks of culture. All cultures were kept in a growth chamber with a 16h photoperiod (50-60 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps) and temperatures of 25ºC (light) and 20ºC (dark) (standard conditions).

Shoot multiplication stage

After 8 weeks of culture, the new shoots obtained in the establishment phase were excised from the original explants and subcultured to produce a shoot multiplication culture. On the basis of our previous experience with adult holm oak material (Martínez & *al.*, 2017a), the multiplication medium used consisted of WPM supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} Sigma agar, and 20 µM silver thiosulphate (STS). Shoots were transferred to 30 x 150 mm culture tubes with 20 ml of fresh medium every two weeks, in a 6-week multiplication cycle, in which proliferation medium was supplemented with 0.1 mg L^{-1} benzyadenine (BA) for the first 2 weeks, 0.05 mg L^{-1} BA for the next 2 weeks, and 0.01 mg L^{-1} BA plus 0.1 mg L^{-1}zeatin for the last 2 weeks. At the end of the 6-week cycle, vigorous shoots longer than 0.5-1.0 cm that had developed from axillary buds were isolated and used for subsequent culture. Subculture on the proliferation media was successively repeated until the number of shoots obtained was sufficient to study the effect of the explant type and the genotype on proliferation rates. The different genotypes in vitro established (each from a different acorn) were named BOA 1 to BOA 13. Genotypes BOA 1 and BOA 5 were the first to in vitro stabilise, and also had the best morphological appearance. Therefore, these genotypes were selected for subsequent experiments.

To investigate the influence of the type of explants on shoot proliferation, apical segments and nodal segments were cut from the shoots longer than 10 mm and cultured on proliferation medium in accordance with 6wk standard multiplication cycle.

To evaluate the influence of genotype on shoot proliferation, shoots from BOA-1 and BOA-5 were cultured on proliferation medium in accordance with 6wk standard multiplication cycle.

Twenty-four shoots were used per each factor (genotype or explant type), and all experiments were carried out in duplicate. The following variables were measured at the end of the 6-week multiplication cycle: the percentage of explants that formed new shoots (responsive explants), the mean number of new shoots (0.5–1.0 cm and > 1 cm in length) per tube, and the mean length of the longest shoot per explant.

Rooting stage

For rooting, shoots 1.0-1.5 cm in length were isolated from multiplication cultures and the basal leaves were stripped. In a first experiment, shoots were immersed in a solution of indole-3-butyric acid (IBA; 1–2 g L^{-1}) for 1 minute, and then they were cultured on Gresshoff & Doy medium (GD, 1972), with macronutrients reduced to one-third strength (1/3 GD).

In a second experiment, shoots were cultured on Murashige & Skoog medium (1962) with half strength macronutrients (½MS) or on WPM with half strength macronutrients (½WPM) supplemented with 3, 5 or 7 mg L^{-1} IBA in combination with 0.1 mg L^{-1} naphthalene-acetic acid (NAA) for 15 days. After treatment with auxin, shoots were transferred to the same medium without auxin for 4 weeks.

In all treatments, sucrose 30 g L^{-1}, Sigma agar 6 g L^{-1} and 20 µM STS were added. Twenty-four shoots were used per treatment, and all experiments were repeated at least twice. The rooting percentage and number of roots per rooted shoot were determined six weeks after the beginning of the experiment.

Statistical analysis

The influence of the genotype and explant type on shoot proliferation was statistically evaluated by a one-way analysis of variance (ANOVA). Factors influencing rooting (mineral medium and auxin concentration) were evaluated by two-way ANOVA. Tests for normality and homogeneity of variance were performed prior to ANOVA, and the least significant difference (LSD) or Dunnett's T3 test ($P \leq 0.05$) was used to compare means in the case of homogeneous or non-homogeneous variances, respectively. The arcsine square-root transformation was applied to proportional data prior to analysis, and the non-transformed data are presented in tables. SPSS for Windows (version 19.0; SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Establishment stage

Germination was only observed in the acorns which were not stored in cold conditions. After incubation

of the acorns in the growth chamber, germination with radicle and shoot emergence occurred in 50% of seeds after 6–12 weeks. The shoot length ranged from 1 to 10 cm (Fig. 1A). Shoots obtained from these germinated seeds were used as initial explants for the *in vitro* establishment of holm oak. Despite applying soft sterilization, the contamination rates of the initial explants *in vitro* cultured were very low (1%) as the seedlings were incubated in the largely sterile environment of a growth chamber. Sprouting buds were obtained on the 13 genotypes introduced *in vitro* (Fig. 1B), although marked differences were observed in the *in vitro* performance. The genotypes BOA-1, BOA-5 and BOA-6 produced the highest proportion of new shoots. The addition of ascorbic acid and transfer of the explants to a different area of the culture medium appears to have been successful in limiting the negative effects of phenolic compounds and other exudates. Nodal segments were more reactive than shoot tip explants (data not shown).

Proliferation stage

Shoots ≥ 0.5 cm produced by the initial cultures were isolated and subcultured following a standard multiplication cycle of 6 weeks. At the end of this period, new shoots that had developed from axillary buds were isolated and used for the next subculture. Only newly developed shoots exhibiting vigorous growth were used in successive subcultures. Subculture on multiplication medium was repeated until the number of shoots obtained was sufficient to enable evaluation of the procedures for improving the shoot proliferation stage. Culture stabilization, defined as uniform and continuous growth of vitro shoot cultures, was slow and took at least 10-12 months. Although proliferation of holm oak shoots was stable (Fig. 1C), shoot multiplication proved difficult owing to the slow growth and development of the axillary buds that emerged. As genotypes BOA-1 and BOA-5 grew most vigorously and were some of the first to stabilize, they were therefore selected for use in subsequent experiments.

As in the initiation step, apex explants did not respond to *in vitro* culture in the proliferation step, both explant types were also evaluated. However, in this step, apex and nodal explants of the two genotypes evaluated respond well on *in vitro* culture, although the total number of shoots was slightly higher on nodal explants. Taking this result into account, both types of explants were routinely used for multiplication.

Decapitation and horizontal culture of explants, as described by Vieitez & *al.* (1994), or the addition of auxin to the proliferation media (NAA or IBA) did not improve the proliferation step (data not shown).

Experiments comparing the proliferation rates of both genotypes were performed. Genotypical differences were observed in relation to the morphology of the developed shoots and the proliferation rates. The proliferation rates were evaluated on basis the percentage of responsive explants, the mean number of new shoots per tube, and the mean length of the longest shoot per explant, although with no statistically significant differences in the three parameters evaluated (Table 1). The genotype BOA-1 yielded the highest numbers of shoots and the longest shoots although without significant differences (Table 1).

Table 1. Effect of genotype on the shoot proliferation of *Quercus ilex*. For each genotype, data represent means ± standard error (SE) of 48 shoots. ns: not significant.

Genotype	Explants forming new shoots (%)	Number of shoots ≥ 0.5 cm	Longest shoot length (mm)
BOA-1	54.2 ± 9.5	2.9 ± 0.2	13.4 ± 1.9
BOA- 5	50.0 ± 5.1	2.6 ± 0.4	8.6 ± 0.4
ANOVA I	ns	ns	ns

Rooting stage

Immersion of the basal ends of shoots from genotype BOA-5 in a solution of IBA (dipped in 1–2 g L^{-1} for 1 minute) was found to be ineffective for root production, with high shoot necrosis observed. Rooting was achieved when shoots explants were cultured in ½MS or ½WPM medium supplemented with 3, 5 or 7 mg L^{-1} IBA plus 0.1 mg L^{-1} NAA for 15 days. Rooting rates were significantly influenced by mineral medium, auxin concentration and their interaction ($P \leq 0.001$). The best results (62.5%) were obtained with 3 mg L^{-1} IBA and ½MS medium. Low rooting frequencies (20.8%) were obtained with 7 mg L^{-1} IBA. The culture medium significantly ($P \leq 0.05$) affected the root length, with 3 mg L^{-1} IBA and ½MS medium yielding higher values. Roots generally began to emerge during the fourth and fifth week after culture on medium without auxin.

Later on, shoots from genotype BOA-1 were cultured on ½ MS with 3 mg L^{-1} IBA plus 0.1 mg L^{-1} NAA for 15 days and subsequently transferred to PGR free medium. After 6 weeks of culture, a rooting rate of 16.67% and root length of 12 mm were obtained.

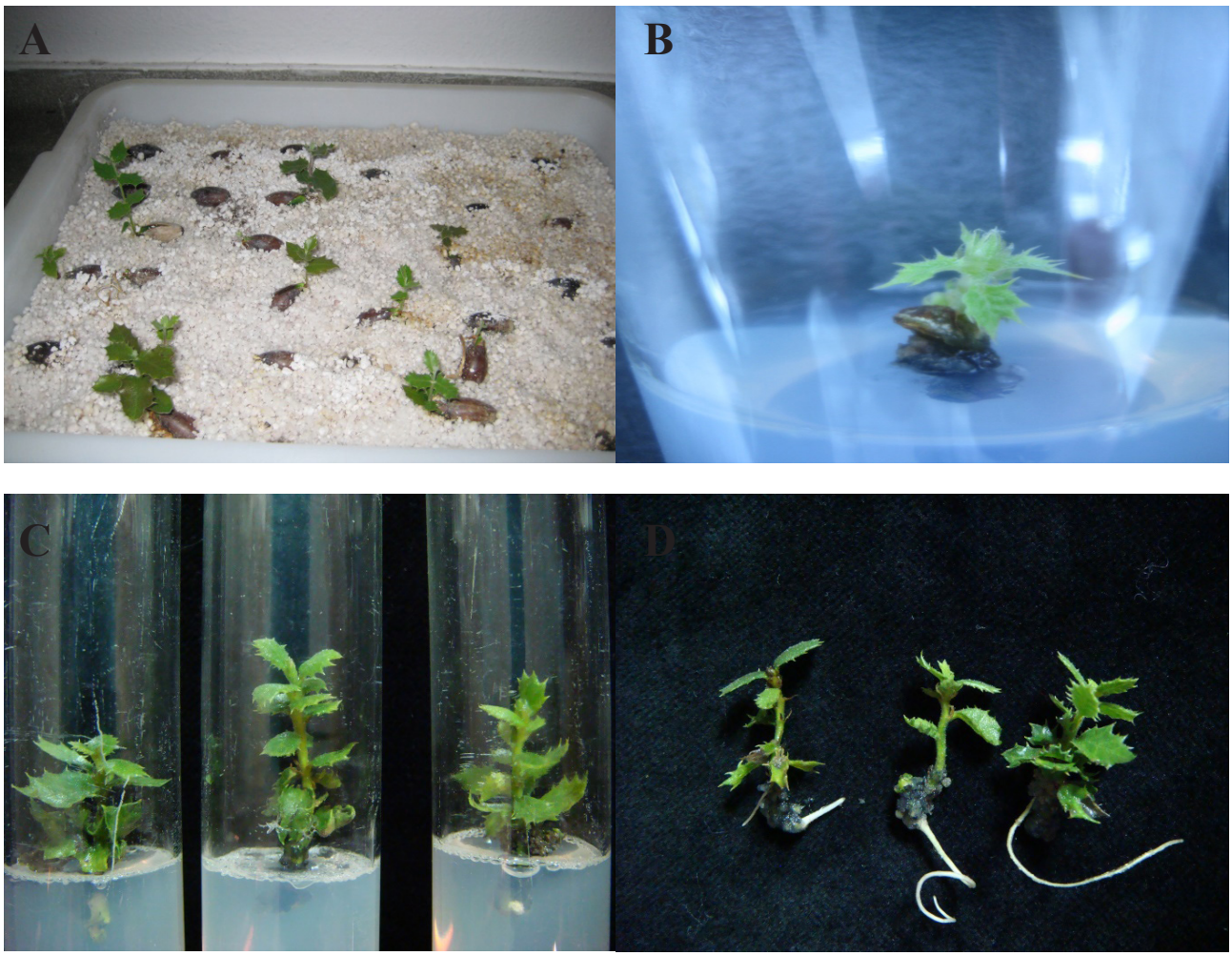

Figure 1. *In vitro* propagation by axillary shoot proliferation of *Quercus ilex*. A: Acorns germinated in the growth chamber. B: Shoot development from nodal explants taken from a shoot obtained after acorn germination. C: Appearance of proliferated shoots after 6-week standard multiplication cycle. D: Root development on shoots treated with IBA 3 mg L^{-1} and NAA 0.1 mg L^{-1} after 8 weeks of culture.

Table 2. Influence of auxin concentration and mineral medium on rooting rates of holm oak in vitro shoots, genotype BOA-5. In each treatment, columns represent means ± SE of 36 explants. Column means followed by different letters differ significantly at P=0.05 level, according to the least significant difference (LSD) or T3 Dunnett test. ns: not significant.

IBA (mg L^{-1})	Rooting (%)		Root length (mm)	
	½MS	½WPM	½MS	½WPM
3	62.5± 1.9a	0.0 ± 0.0e	37.4 ± 3.1	0.0 ± 0.0
5	50.0 ± 0.0a	8.3 ± 0.0d	24.6 ± 3.0	20.0 ± 1.0
7	20.8 ± 1.9b	8.3 ± 0.0d	12.9 ± 1.3	11.2 ± 0.8
Mean			25.1± 5.7a	10.4 ± 4.6b
ANOVA II				
Mineral medium (A)	P ≤ 0.001		P ≤ 0.05	
Auxin (B)	P ≤ 0.001		ns	
A x B	P ≤ 0.001		ns	

Discussion

Seed banks are one of the most extensive *ex situ* conservation procedures. They consist of storing the seeds at low temperature under controlled humidity conditions. Orthodox seeds can be preserved for many years (or even centuries) when they are stored with a low humidity content (generally 3-7% humidity), and at a low temperature (-20ºC). However, numerous woody species have recalcitrant seeds, which are characterized by their rapid loss of viability and lack of tolerance to desiccation, leading to a considerable

reduction in their germination capacity during storage. In this sense, biotechnological techniques, including *in vitro* tissue culture and cryopreservation, provide an opportunity for long-term conservation of tree genetic resources (Corredoira & *al*., 2017). The ultimate goal of forest biotechnology is the clonal propagation of mature trees, elite genotypes or cultivars, threatened tree species or genotypes with known disease or pest resistance (Pence, 1999; Pijut & *al.*, 2011). Indeed, axillary shoot proliferation has been successfully applied to the conservation of several endangered, rare, threatened of extinction, or endemic species (Sudha & *al.*, 1998; Pania & *al.*, 2000; Almeida & *al.*, 2005). Recently, San José & *al.* (2017) has defined a procedure for *ex situ* conservation through *in vitro* techniques on *Quercus lusitanica*, an endangered species. In the case of the holm oak, which is severely affected by *la seca*, difficult to propagate vegetatively and with recalcitrant seeds that cannot be preserved by the methods conventionally used in traditional seed banks, micropropagation procedures must be developed to enable propagation and conservation of the species.

A critical factor on the in vitro establishment of holm oak is the sterilisation step, as we found that culture initiation was only possible when low-level disinfection protocols were used. Holm oak material showed great sensitivity to sterilising agents, and procedures routinely applied for the sterilisation of shoots derived from other *Quercus* species (Vieitez & *al.,* 1994; Vieitez & *al.,* 2009) have been reported to have a lethal effect in holm oak shoots, with response rates of 0%.

The achievement of uniform and continuous *in vitro* shoot growth is highly problematical for woody species with a strongly episodic growth habit (McCown, 2000). In this respect, *Quercus* species are typical of woody perennials characterized by strong episodic flushes during the growing season (Vieitez & *al.*, 2012). The difficulties related to *in vitro* culture of these species are attributed to the inability to reach the stabilization stage, during which uniform and continuous shoot growth is displayed (Vieitez & *al.*, 2009). Nevertheless, the procedure used in the present study has enabled the establishment and proliferation of two different holm oak genotypes by *in vitro* culture of explants derived from juvenile material.

The micropropagation capacity can differ enormously depending on the genotype (Monteuuis, 2016). The findings of the present study showed that different genotypes were able to be established *in vitro*; however, differences in the responses of the different genotypes were observed during the in vitro establishment and proliferation stage. This genotypic effect has been reported for other oak species, such as pedunculate oak (Meier-Dinkel & *al.*, 1993; Juncker & Favre, 1989) and American oak species (Vieitez & *al.*, 2009). Although genotype has a strong influence on the propagation of holm oak, the protocol described in the present study could be applied to different genotypes to produce elongated shoots of healthy appearance.

Axillary shoot multiplication can depend of the explant type used (San José & *al.*, 1990; Vieitez & *al.*, 2007), however in the present study this fact did not significantly affect the proliferation, with satisfactory multiplication rates obtained with apical and nodal explants. Similarly, the proliferation of shoots established from mature material of holm oak is not influenced by the explant type (Martínez & *al.*, 2017a).

The rhizogenic capacity of woody species generally decreases as the tree ages but occurs at a particularly early age in holm oak. L'Helgoual'Ch & Espagnac (1987) demonstrated that at age 3 months, some 70% of the shoots rooted, whereas one month later the rate of rhizogenesis did not exceed 30%. Despite the difficulty in rooting displayed by holm oak, acceptable rooting rates were achieved in the present study by culturing the shoots in medium containing 3 or 5 mg L^{-1} IBA plus 0.1 mg L^{-1} NAA for 15 days on ½MS medium, with subsequent transfer to auxin-free medium. IBA is the auxin most widely used for rooting micropropagated shoots of *Quercus* spp, either by adding it to the medium for different lengths of time or by dipping the basal ends in concentrated solutions of the auxin (Vieitez & *al.,* 2012). In juvenile material of holm oak, Liñán & *al.* (2011) obtained rooting rates of between 28 and 40% after dipping the basal ends of shoots in IBA solutions. However, in our case dipping the basal ends in IBA these treatments were found to be totally ineffective, and rooting was only obtained by adding IBA and NAA to the culture medium for 15 days. The incorporation of both auxins has also been reported by Ostrolucka & *al.* (2007) in different species of *Quercus*.

Mineral medium also affected the rooting capacity of holm oak. For rooting of oak species, shoots were usually cultured on the same mineral medium as the proliferation step, but frequently at levels less than full-strength (Vieitez & *al.*, 2012). However, in holm oak, WPM medium used in the proliferation, but reduced to half in the rooting, obtained poorer results than ½ MS. Reduced salt strength of MS containing a low concentration of IBA has also a positive effect on shoot rooting of *Dionaea muscipula* (Jang & *al.*, 2003). Likewise, in the hybrid *Prunus persica* x *P. amygdalus* MS medium reduced to half the normal value also increased the rooting frequencies (Fotopoulos & Sotiropoulos, 2005).

Finally, genotypic differences were observed in the rooting response of holm oak. Xing & *al.* (1997) also obtained genotype differences in shoot rooting of American chestnut.

Conclusions

The results obtained in the present study demonstrate that *in vitro* micropropagation in *Quercus ilex* is possible from juvenile material derived from germination of acorns, despite holm oak being a woody plant with episodic growth habit and consequently difficult *in vitro* establishment. The above-mentioned findings indicate that the procedure

developed could potentially be used for large-scale production of holm oak plants and for the conservation of this important tree species. However, *in vitro* culture of holm oak is highly dependent of the genotype, affecting both the stabilizing phase as well as that of the rooting. Further investigations are necessary to optimize micropropagation conditions in order that a greater number of genotypes may be established and rooted.

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